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13. ABSTRACT *(Maximum 200 Words)*

Diindolylmethane (DIM) is formed by acid catalyzed dimerization of indole-3-carbinol, and both compounds inhibit formation and/or growth of mammary tumors in rodents. In this study, we have investigated the aryl hydrocarbon receptor (AhR) agonist activity and inhibitory AhR-estrogen receptor crosstalk induced by the following methyl-substituted DIMs: 1,1'-dimethyl-, 2,2'-dimethyl-, 5,5'-dimethyl-, 6,6'-dimethyl-, and 7,7'-dimethylDIM and 1,1',2,2'-tetramethylDIM. The six compounds exhibited minimal to non-detectable AhR agonist or antagonist activities associated with CYP1A1 induction. In contrast, the methyl-substituted DIMs inhibited estrogen-induced T47D human breast cancer cell growth. The antitumorigenic activity of these compounds was examined in 7,12-dimethylbenz[a]anthracene-induced rat mammary tumor model in which the DIM analogs were orally administered (by gavage in corn oil) at a dose of 1 mg/kg/ every second day (X10). 1,1'-DimethylDIM, 5,5'-dimethylDIM and 1,1',2,2'-tetramethylDIM significantly inhibited mammary tumor growth and this was not accompanied by changes in organ/body weights or histopathology. These studies demonstrate that methyl-substituted DIMs are selective AhR modulators (SAhRMs) with potential for clinical treatment of breast cancer.

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Table of Contents

Introduction

TCDD induces a broad spectrum of toxic response and modulates several endocrine signaling pathways [1]. For example, TCDD is a potent antiestrogen and inhibits E2-induced gene expression and growth in the rodent uterus, human breast cancer cells, and rodent mammary tumors [reviewed in 2-5]. Research in our laboratory has focused on delineating the mechanisms of inhibitory AhR-ER crosstalk and development of relatively nontoxic selective AhR modulators (SAhRMs) for treatment of breast cancer [3]. Previous studies show that alternate substituted alkyl polychlorinated dibenzofurans (PCDFs), DIM, and halogen-substituted DIMs are relatively nontoxic SAhRMs that inhibit mammary tumor growth in carcinogen-induced female Sprague-Dawley rats [6-8]. This study also shows that dimethyl-substituted DIMs are also antiestrogenic in human breast cancer cells and the rodent mammary tumor model, and thereby represent additional SAhRMs that can be used for AhR-based treatment of breast cancer.

Body

Cell proliferation studies

Initial screening of the methyl-substituted DIMs (Fig. 1) used cell proliferation assays in T47D human breast cancer cells that are responsive to the mitogenic effects of E2 but are also inhibited by cotreatment with AhR agonists [8]. The results illustrated in Figure 2 summarize the effects of six methyl-substituted DIMs alone and in combination with E2 on growth of T47D cells. The compounds alone had minimal effects on growth of the cells using concentrations of 0.1, 1.0, 5.0 and 10 μM. In cotreatment studies, 5 and 10 μM concentrations of all methyl-substituted DIMs inhibited E2-induced proliferation of T47D cells and the most active compound significantly inhibited growth at concentrations of 0.1 (2,2'-diMeDIM and 5,5'-diMeDIM) or 1.0 μM (1,1'-diMeDIM). The results indicate that methyl-substituted DIMs exhibit antiestrogenic activity and this included compounds substituted at the hetero N atom, and in both rings of the indole moiety.

Methyl-substituted DIMs as AhR agonists

Initial studies showed that all six methyl-substituted DIMs did not induce CYP1A1-dependent EROD activity in T47D human breast cancer cells (Fig. 3); however, in cells cotreated with these compounds plus DIM, there was complete inhibition of induced EROD activity. The apparent AhR antagonist activity of the dimethylDIMs in this assay is ambiguous since DIMs directly interact with CYP1A1 and are potent inhibitors of CYP1A1-dependent activity [9]. The AhR agonist/antagonist activity of these compounds was further investigated in T47D cells transfected with Ah-responsive pRNH11c containing the –1142 to +2434 region of the human CYP1A1 gene promoter fused to the bacterial CAT reporter gene (Fig. 4). The results show that at concentrations as high as 10 μM, the substituted DIMs did not induce CAT activity and in cells cotreated with TCDD plus tetra-dimethylDIMs, CAT activity induced by TCDD was not inhibited. These results indicate that 10 μM concentrations of the methyl-substituted DIMs exhibited minimal AhR agonist or antgonist activity associated with induction of CYP1A1, whereas these same compounds were active as antiestrogens in this cell lines (Fig. 2).

***In Vivo* antiestrogenic activities of tetra-dimethylDIMs**

The results in Figure 5 summarize the antitumorigenic activity of the four methyl-substituted DIMs using the DMBA-induced rat mammary tumor model. Fifty day-old female Sprague-Dawley rats were initiated with DMBA, and within 6 to 12 weeks, mammary tumors developed. Animals were then treated with corn oil (control) or DIM analogs in corn oil every other day for twenty days and sacrificed on day 21. The dose used for all compounds was 1 mg/kg, and the results show that 5,5'-dimethylDIM, 2,2'-dimethylDIM and 1,1',2,2'-tetramethylDIM significantly inhibited tumor growth (Fig. 5) but did not alter organ weights (Table 1). In contrast, 1,1'-dimethylDIM did not significantly inhibit tumor growth at the dose used in the experiment. Thus, three out of the four methyl-substituted DIMs were antitumorigenic at the 1.0 mg/kg dose and were more active than DIM which inhibited tumor growth at the 5 mg/kg but not 1 mg/kg dose levels [7].

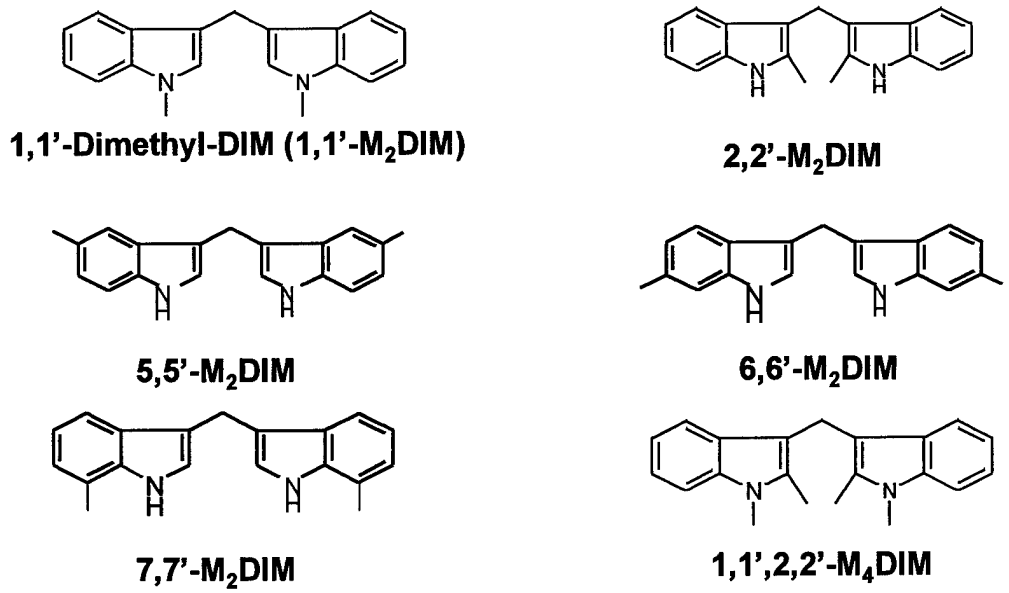

Fig.1. Structures of methyl-substituted diindolylmethanes.

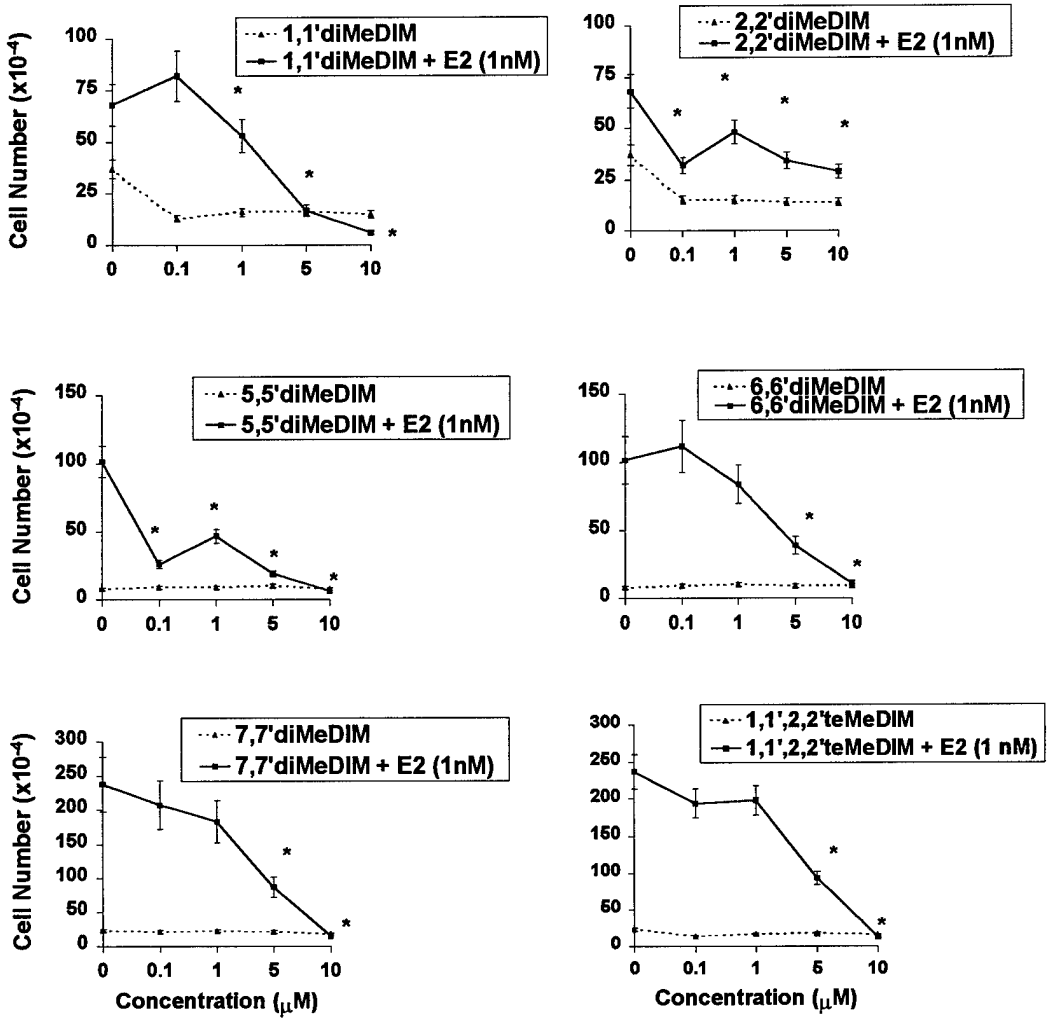

Fig. 2. Estrogenic and antiestrogenic activities of alkyl-substituted DIMs in T47D cells.
Effects of alkyl-substituted DIMs (100 nM to 10 uM) alone or in combination with 1 nM E2 on T47D cell proliferation were determined. Results are the average of 3 replicate experiments expressed ± standard error.
* Significant inhibition ($p<0.05$) of E2-induced cell proliferation.

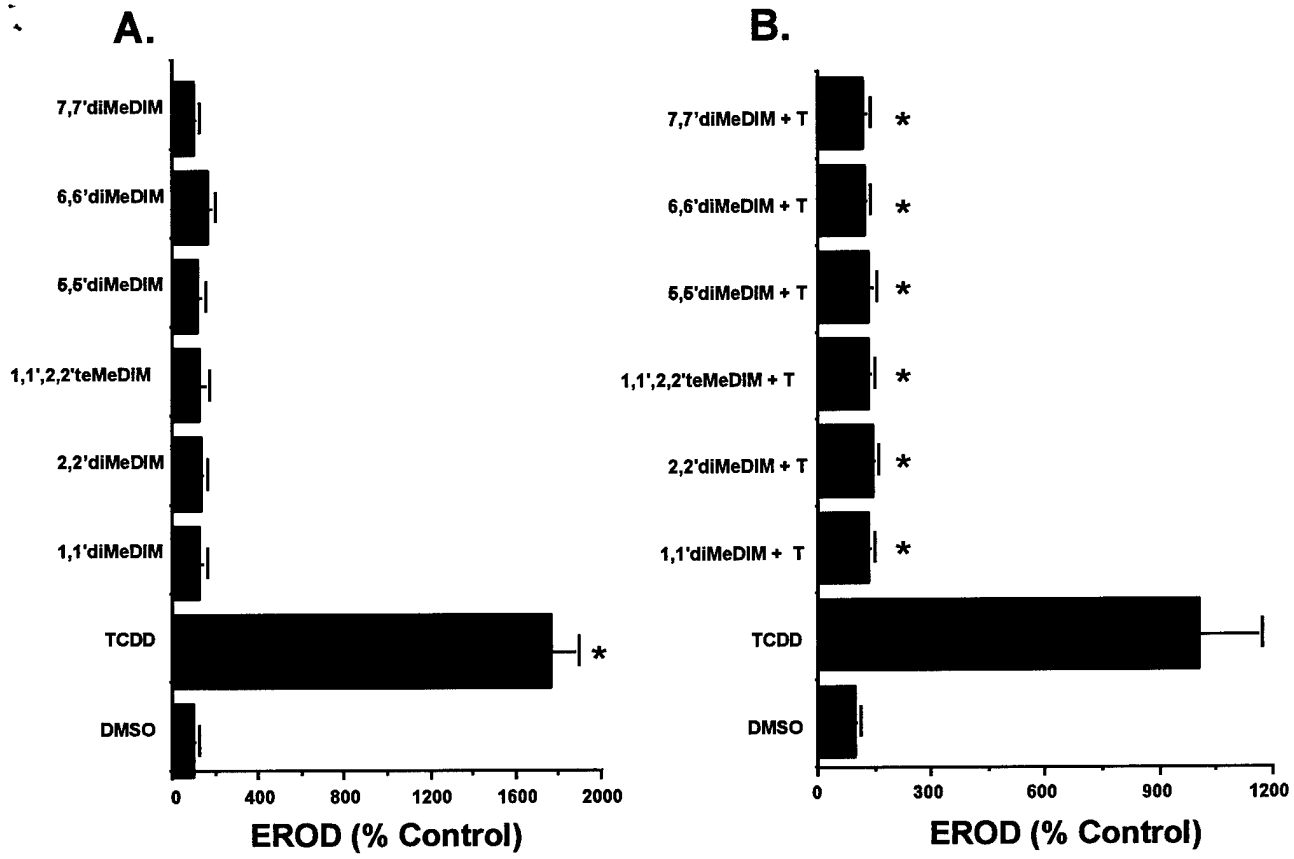

Fig. 3. EROD induction (A) and inhibition of TCDD-induced EROD activity (B) by alkyl-substituted DIMs in T47D cells. Cells were treated with DMSO vehicle, 1 nM TCDD, 100 uM alkyl-substituted DIMs (50 and 10 mM not shown) or cotreated with 1 nM TCDD plus 10 uM alkyl-substituted DIMs and EROD activities were determined fluorimetrically. Results are the average of 3 replicate experiments expressed percent control ± standard error.
(A) * Significant induction ($P<0.05$) of EROD activity compared to control experiment.
(B) * Significant inhibition ($P<0.05$) of TCDD-induced EROD activity.

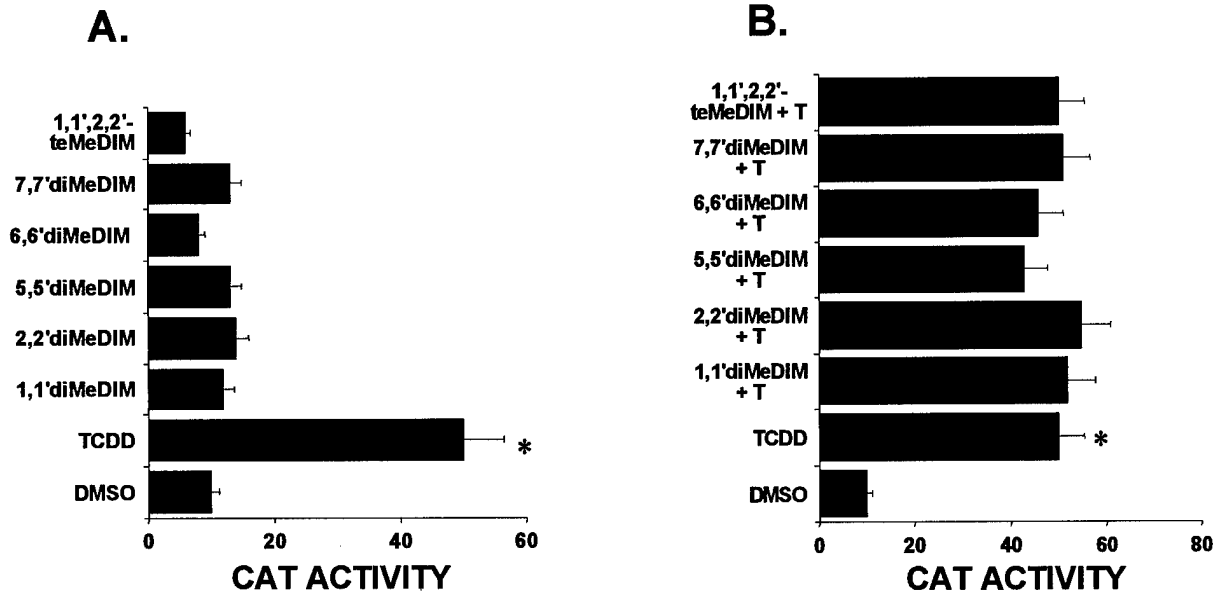

Fig. 4. AhR agonist (A) and antagonist (B) activities of alkyl-substituted DIMs in T47D cells transfected with pRNH11c. Cells were treated with DMSO (control), TCDD (T) (1 nM) and compounds (10 uM) alone or in combination with TCDD and CAT activities were determined. Results are expressed as means ± S.E. for three separate experiments for each treatment group.
* Significant induction ($P<0.05$) of CAT activity.

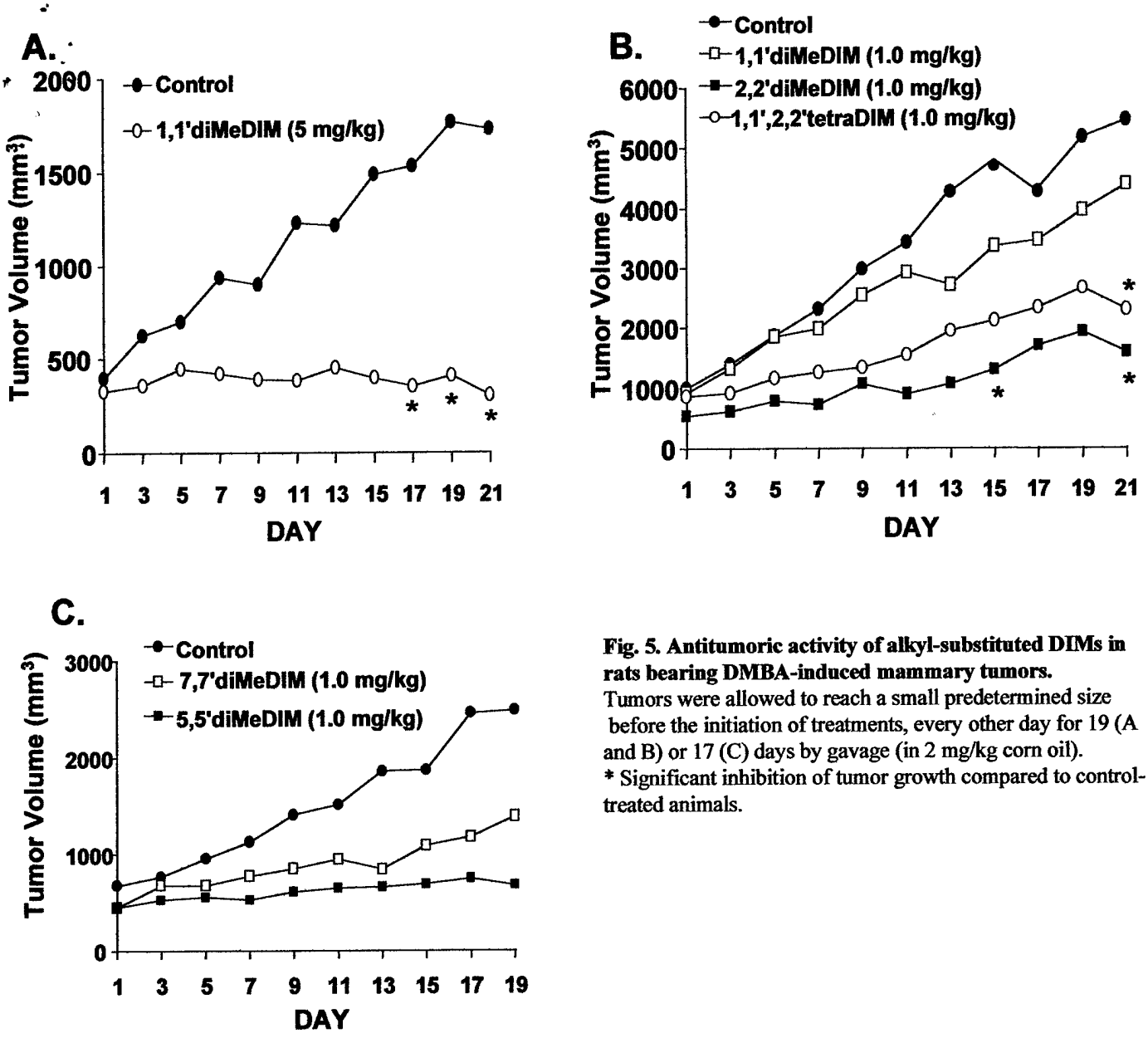

Fig. 5. Antitumoric activity of alkyl-substituted DIMs in rats bearing DMBA-induced mammary tumors.
Tumors were allowed to reach a small predetermined size before the initiation of treatments, every other day for 19 (A and B) or 17 (C) days by gavage (in 2 mg/kg corn oil).
* Significant inhibition of tumor growth compared to control-treated animals.

Table 1. Effects of treatment on tumor growth and and organ weights.

	Final tumor volume (%control)	Rate of tumor growth (mm^3/day)	Liver wt (% body wt)	Uterine wt (% body wt)	Heart wt (% body wt)	Spleen wt (% body wt)	Kidney wt (% body wt)
Control	100 ± 24	69 ± 4	3.4 ± 0.2	0.19 ± 0.01	0.38 ± 0.01	0.30 ± 0.02	0.34 ± 0.17
1,1'diMeDIM	18 ± 5*	0 ± 2*	3.0 ± 0.2	0.20 ± 0.02	0.39 ± 0.02	0.25 ± 0.01	0.35 ± 0.02
Control	100 ± 31	233 ± 15	3.8 ± 0.3	0.24 ± 0.07	0.41 ± 0.02	0.41 ± 0.06	0.41 0.02
2,2'diMeDIM	75 ± 33	65 ± 8*	3.3 ± 0.1	0.16 ± 0.01	0.40 ± 0.02	0.29 ± 0.3	0.38 ± 0.02
1,1'diMeDIM	29 ± 9*	162 ± 8	3.7 ± 0.2	0.19 ± 0.02	0.41 ± 0.02	0.36 ± 0.02	0.52 ± 0.01
1,1',2,2'teMeDIM	40 ± 22*	91 ± 7*	3.6 ± 0.2	0.17 ± 0.02	0.41 ± 0.02	0.33 ± 0.06	0.37 ± 0.01
Control	100 49	106 ± 6	3.7 ± 0.2	0.27 ± 0.05	0.41 ± 0.01	0.22 ± 0.02	0.85 ± 0.02
5,5'diMeDIM	27 ± 24	43 ± 5*	4.3 ± 0.2	0.23 ± 0.05	0.41 ± 0.01	0.25 ± 0.01	0.91 ± 0.05
7,7'diMeDIM	56 ± 10	14 ± 2*	4.1 ± 0.2	0.03 ± 0.04	0.44 ± 0.01	0.30 ± 0.02	0.90 ± 0.04

Treatment of mammary-tumor bearing rats with alkyl-substituted DIMs did not affect organ weights.
No gross pathology was observed in the liver, spleen, kidney, heart or uterus of treated animals.

* Significantly different ($p < 0.05$) from controls.

Key Research Accomplishments

·DialkylDIMs inhibit E2-induced responses in T47D and MCF7 human breast cancer cells.

·These compounds interact with the AhR but do not induce CYP1A1(a surrogate for toxicity) or inhibit TCDD-induced CYP1A1 expression.

·Inhibition of EROD activity by methyl-substituted DIMs is probably due to direct binding to the catalytic site of CYP1A1.

·Methyl-substituted DIMs have minimal antiestrogenic effects in the immature mouse uterus.

·Some of these compounds are potent inhibitors of mammary tumor growth in carcinogen induced female Sprague-Dawley rats.

Reportable Outcomes

(a) Manuscripts, abstracts, presentations

Ramamoorthy, K., McDougal, A. and Safe, S. H. Structure-Ah receptor agonist/binding activity relationships of various chlorine-substituted diindolylmethane compounds. *Organohalogen Compounds* 42:363-367, 1999.

McDougal, A., Sethi-Gupta, M., Ramamoorthy, K., Sun, G. and Safe, S. Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symmetrical dihalo-substituted analogs of diindolylmethane. *Cancer letts.* 151:169-179, 2000.

M.D. Morrow, A.J. McDougal and S. Safe. Methylene-substituted 1,1'-dimethyldiindolylmethane analogs as inhibitors of carcinogen-induced mammary tumor growth in rodents. *Toxicologist* 54, 1020, 2000.

Andrew McDougal, Mona Sethi Gupta, Derek Morrow, Kavita Ramamoorthy, Jeong-Eun Lee, and Stephen H. Safe. Metyhl-substituted diindolylmethanes as inhibitors of estrogen-induced responses and mammary tumor growth. *Cancer letts.,* in preparation (2000).

(b) Patents/licenses applied for or issused

DIMs and C-Substituted DIMs as Anticancer Agents
Provisional Patent submitted.

(c) Degrees

Mona Sethi-Gupta - Ph.D (2000)
"Mechanistic Studies of Xenobiotic & Natural Compounds That Modulate Estrogen Receptor and Aryl Hydrocarbon Receptor Signaling Pathways.

(d) Cell lines/serum

No new lines developed.

(e) Informatics

None.

(f) Funding applied for

None.

(g) Employment/research opportunities

Mona Sethi-Gupta - Postdoctoral Fellowship, Medical College of Virginia, Richmond, VA.

Conclusions

Like the dihaloDIMs, methylsubstituted analogs also exhibit both antiestrogenic and antitumorigenic activity and represent the first group of active SAhRMs that do not contain halogen substitution. These studies demonstrate that methyl-substituted DIMs are selective AhR modulators (SAhRMs) with potential for clinical treatment of breast cancer.

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Appendices

Enclosed.

Structure-Ah Receptor Agonist/Binding Activity Relationships of Various Chlorine-Substituted Diindolylmethane Compounds

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Introduction

Diindolylmethane (DIM) is the dimerization product of indole-3-carbinol (I3C), an antitumorigenic compound found in cruciferous vegetables. DIM has previously been shown to inhibit 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumors, as well as 17β-estradiol (E_2)-induced cell proliferation in breast cancer cells. Research in this laboratory has shown that the antitumorigenic and antiestrogenic responses are mediated via the aryl hydrocarbon receptor (AhR) (1,2). DIM and several chlorine-substituted analogs were examined in this study for antiestrogenic and antitumorigenic activity *in vivo*. The female B6C3F1 mouse uterine model was used for *in vivo* antiestrogenicity studies, and effects of E_2 and E_2 + substituted DIMs on progesterone receptor levels, uterine peroxidase activity and uterine wet weight were determined. The 7,12-dimethylbenzanthracene (DMBA) rat mammary tumor model was used to determine antitumorigenic activity of substituted DIMs. Results indicate that 4,4'-Cl_2DIM, 5,5'-Cl_2DIM and 6,6'-Cl_2DIM competitively bound rat AhR, however only 5,5'- and 6,6'-Cl_2DIM were antiestrogenic in the immature mouse uterus. 4,4'-Cl_2DIM and 6,6'-Cl_2DIM but not 5,5'-Cl_2DIM exhibited antitumorigenic activity in the female rat.

Materials and Methods

Compounds. The following substituted DIMs were synthesized in the laboratory: 4,4'-dichlorodiindolylmethane (4,4'-Cl_2DIM), 5,5'-dichlorodiindolylmethane (5,5'-Cl_2DIM), and 6,6'-dichlorodiindolylmethane (6,6'-Cl_2DIM).

Animals. Twenty-one day-old B6C3F1 female mice were purchased from Jackson Laboratories and housed 6-9 per cage with *ad libitum* access to food and water. DIM and substituted analogs was dissolved in corn oil with slight warming and the total dose divided into 3 daily administrations. Animals were divided into 3 groups of 6-8 animals each and dosed for 3 days on days 21, 22 and 23. One group received vehicle control corn oil (50 μl) by gavage. The second group received 0.02 μg/day E_2 (in corn oil) by i.p. injection. The third group received 100 mg/kg DIM or substituted analog by gavage plus 0.02 μg/day E_2 by i.p. injection. The doses of E_2 were the minimal effective

dose which significantly induced the 3 uterine responses of interest. Animals were killed by carbon dioxide asphyxiation 20 h after the last treatment and uteri were quickly removed, cleaned of connective tissue, weighed, nicked, and blotted. The uteri were then bisected into right and left halves, each half containing an entire uterine horn.

Progesterone Receptor Binding Assay (PR). PR binding was carried out as previously described (3). Analysis was conducted on pooled uteri from each treatment group and levels are reported in fmol per uterus. Assays were carried out in triplicate and results are given as mean ± standard error.

Uterine Peroxidase Assay (UPO). UPO activity was carried out as previously described (3). Analysis was conducted on pooled uteri from each treatment group and enzyme activity was expressed per uterus. The assay was carried out in triplicate and results are given as mean ± standard error.

Cytosolic AhR Binding Assay. Male Sprague-Dawley rats (4-5 weeks old) were sacrificed by CO_2 asphyxiation and cervical dislocation and livers were perfused with ice-cold HEGD [25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol (v/v)] buffer. Livers were homogenized in HEGD (3 ml/g tissue) using a Brinkman/Polytron homogenizer. Homogenates were centrifuged at 10,000 g for 10 min (4°C) and the resulting supernatant was centrifuged at 105,000 g for 1 hr (4°C). The resulting pellet was resuspended in 7-9 ml HEGD buffer and protein concentration measured by the method of Bradford (1976). AhR binding was measured using the hydroxylapatite (HAP) assay. HAP was washed twice with HEGD buffer (pH 7.4) and then resuspended in 2 vol of HEGD buffer. Rat hepatic cytosol (3.0 mg/ml) was incubated with 3 nM [^{3}H]TCDD, 3 nM [^{3}H]TCDD plus a 200 fold excess of unlabelled TCDF or [^{3}H]TCDD plus varying concentrations of DIM/substituted analogs for 2 hr at 20°C in a shaking water bath. The incubation mixture was then added to 100 µl of the HAP suspension in a disposable 13 X 100 mm glass test tube and further incubated for 30 min at 0 - 4°C with gentle shaking every 10 min. HEGD buffer (1.0 ml) containing 0.5% (v/v) Tween 80 was then added and the tubes were vortexed and centrifuged at 1500 rpm for 5 min. The supernatant was decanted and washed (3x) and ethanol (1.0 ml) was added to the HAP pellet . The tube was vortexed and the contents removed with a Pasteur pipette and the bound [^{3}H]TCDD was determined by liquid scintillation counting.

Tumor Studies. Fifty day-old female virgin Sprague-Dawley rats were obtained from Harlan (Houston) and allowed to acclimate for 5 days. On day 54, each rat received 20 mg 7,12-dimethylbenzanthracene dissolved in 0.5 ml corn oil. After the formation of tumors (750 - 1250 mm^3), rats were dosed every other day by oral gavage for 20 days (10 doses) with either corn oil (vehicle) or substituted DIM (1 or 5 mg/kg). Tumor sizes were measured every other day and rats were euthanized on day 21.

Results and Discussion

1. At a dose of 100 mg/kg, both 5,5'-Cl_2DIM and 6,6'-Cl_2DIM significantly inhibited several estrogen-induced markers in the immature mouse uterus (Figure 1):

a) 5,5'-Cl_2DIM significantly inhibited PR and UPO activity, 62% and 41%, respectively.
b) 6,6'-Cl_2DIM significantly inhibited uterine wet weight, PR and UPO activity, 16%, 25% and 67%, respectively.
c) 4,4'-C_2lDIM did not significantly inhibit any of the uterine markers.
d) No compound significantly induced hepatic cytochrome P450 1A1 activity.

2. Competitive AhR binding assays were performed with rat liver cytosol. All substituted DIMs displaced 3 nM [^{3}H]TCDD from the AhR, indicating competitive binding to the receptor. Binding curves for the compounds are shown in Figure 1. EC50 values are:

$$4,4'\text{-}Cl_2\text{DIM} = 6.89 \times 10^{-10}\ \text{M}$$
$$5,5'\text{-}Cl_2\text{DIM} = 8.67 \times 10^{-9}\ \text{M}$$
$$6,6'\text{-}Cl_2\text{DIM} = 1.99 \times 10^{-8}\ \text{M}$$

indicating a rank order potency as follows: 4,4'-Cl_2DIM > 5,5'-Cl_2DIM > 6,6'-Cl_2DIM. None of the compounds competitively bound the estrogen receptor (data not shown).

3. Studies in the DMBA-induced mammary tumor model indicate that 4,4'-Cl_2DIM and 6,6'-Cl_2DIM were significantly antitumorigenic at a dose of 1.0 mg/kg/2d. 5,5'-Cl_2DIM was not antitumorigenic at 1.0 or 5.0 mg/kg/2d. None of the compounds induced hepatic cytochrome P450 1A1 activity.

4. Data presented herein suggest that chlorine-substituted DIM analogs exhibit antiestrogenic and antitumorigenic activity. Of the analogs tested, 6,6'-Cl_2DIM was the most potent antiestrogen/antitumorigen, inhibiting all three estrogen-induced markers in the immature mouse uterine model and significantly inhibiting tumor growth at a dose as low as 1.0 mg/kg/2d.

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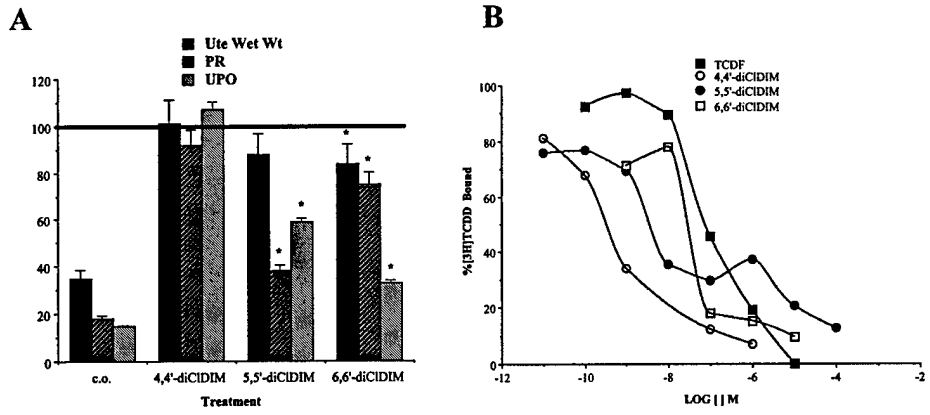

Figure 1. (A) Antiestrogenic activity of chlorine-substituted DIM analogs in the immature mouse uterine model. *Significantly antiestrogenic ($p < 0.05$). (B) Competitive AhR binding activity of chlorine-substituted DIM analogs.

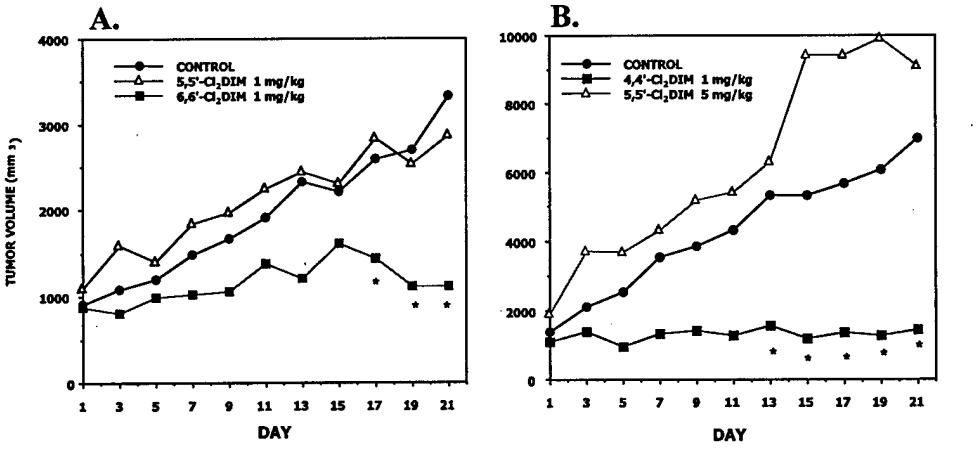

Figure 2. (A) Antitumorigenic activity of 5,5'-Cl_2DIM and 6,6'-Cl_2DIM at 1.0 mg/kg/day in the DMBA-induced rat mammary tumor model. (B) 4,4'-Cl_2DIM at 1.0 mg/kg/day and 5,5'-Cl_2DIM at 5.0 mg/kg/day in the DMBA-induced rat mammary tumor model.

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Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symmetrical dihalo-substituted analogs of diindolylmethane

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Abstract

This study investigates the antiestrogenic/estrogenic and antitumorigenic activities of the following diindolylmethane (DIM) derivatives: 4,4′-dichloro-, 5,5′-dichloro-, 6,6′-dichloro-, 5,5′-dibromo-, 5,5′-difluoro- and 5,5′-dichloro-2,2′-dimethylDIM. E2-induced proliferation of T47D breast cancer cells was significantly inhibited (>90%) by the haloDIMs at concentrations of 5 or 10 μM, and only 4,4′-dichloroDIM alone increased cell proliferation. With the exception of 5,5′-difluoroDIM, the remaining compounds also inhibited E2-induced growth of MCF-7 human breast cancer cells. DihaloDIMs (100 mg/kg/day × 3) were not estrogenic in the immature female B6C3F1 mouse uterus; however, in animals co-treated with E2 (0.02 μg/mouse), 5,5′-dichloro- and 6,6′-dichloroDIM inhibited uterine progesterone receptor (PR) binding and uterine peroxidase activity, whereas 5,5′-dichloro- and 5,5′-dichloro-2,2′-dimethylDIM inhibited only the latter response. The antitumorigenic activities of the dihaloDIMs were determined by their inhibition of carcinogen-induced mammary tumor growth in female Sprague–Dawley rats. 4,4′-Dichloro-, 5,5′-dibromo- and 6,6′-dichloroDIM, significantly inhibited mammary tumor growth at doses of 1 mg/kg every second day, and no significant changes in organ weights or liver and kidney histopathology were observed. These three compounds were more active than DIM in the same in vivo assay.

Keywords: Dihalodims; Antitumorigenic; Ah receptor

1. Introduction

Lifetime exposure to estrogens is an important risk factor for breast cancer in women; however, the precise role of estrogens in development of this disease is complex [1]. Estrogens alone or in combination with other mitogens such as growth factors promote breast cancer cell growth and hormonal estrogens could function as tumor promoters [2,3]. In addition, there is evidence that estrogenic metabolites, including 16α-hydroxy-17β-estradiol and the 2- and 4-catechol estrogens, may play a role in tumor initiation and other steps in mammary carcinogenesis [2–6]. Antiestrogenic drugs, such as tamoxifen and other estrogen receptor (ER) agonists, have been extensively developed for treatment of hormone-dependent mammary tumors, and these compounds primarily act by blocking ER action [7,8].

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In addition to direct-acting antiestrogens, several other classes of drugs inhibit estrogen-induced responses. For example, aromatase inhibitors, which block metabolic conversion of androgens to 17β-estradiol (E2) and estrone, have been utilized for treatment of hormone-dependent tumors by decreasing levels of hormonal estrogens [9]. Vitamin D analogs and retinoids are being developed for treatment of mammary cancer. These compounds act through inhibitory crosstalk between vitamin D/retinoid receptors and ER signaling pathways [10–12]. Aryl hydrocarbon receptor (AhR) agonists also inhibit E2-induced responses in the rodent uterus and mammary gland and in human breast cancer cells (reviewed in Refs. [13–15]). For example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent AhR agonist, inhibits: (a) age-dependent formation of mammary and uterine tumors in female Sprague–Dawley rats, (b) carcinogen-induced mammary tumor growth in rats, and (c) mammary tumor growth in athymic mice bearing MCF-7 cell xenografts [16–19]. Research in this laboratory has also identified relatively non-toxic AhR agonists that act as antiestrogens and inhibit mammary tumor growth, and these include alternate-substituted (1,3,6,8- or 2,4,6,8-) polychlorinated dibenzofurans (PCDFs) and diindolylmethane (DIM) [20–22]. DIM bound to the AhR and induced formation of the nuclear AhR complex in MCF-7 human breast cancer cells [21] and also inhibited E2-induced breast cancer cell proliferation and growth of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumors in female Sprague–Dawley rats at doses of 5 mg/kg every 2 days.

A series of six symmetrical dihalo-DIMs, including 4,4′-dichloro-, 5,5′-dichloro-, 5,5′-dichloro-2,2′-dimethyl-, 6,6′-dichloro-, 5,5′-dibromo- and 5,5′-difluoroDIM (Fig. 1) were synthesized and their inhibitory activities were determined in estrogen-responsive assays in breast cancer cells, the mouse uterus, and DMBA-induced rat mammary tumors. Three compounds, namely 4,4′-dichloro-, 6,6′-dichloro- and 5,5′-dibromoDIM, inhibited mammary tumor growth in vivo at a dose of 1 mg/kg every second day. Changes in organ or body weights or induction of hepatic CYP1A1-dependent microsomal ethoxyresorufin *O*-deethylase (EROD) activity were not observed. These results indicate that some dihalo-DIMs were significantly more active than DIM [21]

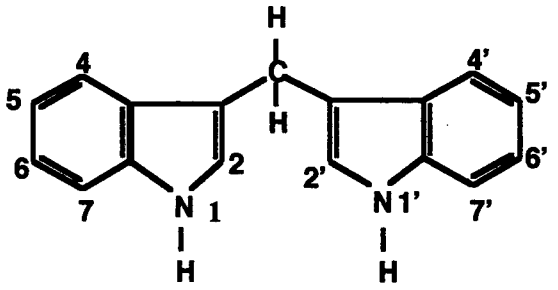

Fig. 1. Structures of symmetrical dihaloDIMs used in this study.

as inhibitors of E2-induced cell proliferation and tumor growth. Their antitumorigenic potencies in other hormone-dependent and -independent tumors are currently being investigated.

2. Materials and methods

2.1. Chemicals and biochemicals

The following substituted indoles were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used to prepare the substituted DIMs: 4-chloroindole, 5-chloroindole, 6-chloroindole, 5-fluoroindole, 5-bromoindole, and 2-methyl-5-chloroindole. A common synthetic route was utilized for preparation of all halo-substituted DIMs. Dimethylformamide (2.9 ml; Aldrich Chemical Co.) was cooled to 0°C in an ice-salt bath and phosphorus oxychloride (0.86 ml; Aldrich Chemical Co.) was added slowly over 30 min. The substituted indole (8.6 mmol) in 1.0 ml dimethylformamide was added slowly to the cooled solution over a period of 10 min and the resulting slurry was then heated at 35°C for 60–90 min until the clear yellow solution became a yellowish paste. The thick slurry was added to 1 ml of water and ice, and 10 ml of aqueous potassium hydroxide solution (3.75 g KOH) was slowly added to the reaction mixture (30 min), which was then heated to the boiling point and cooled to 0°C. The precipitated halo-substituted indole-3-carboxaldehyde was filtered, washed with water, and air dried. The overall yields were 90–100% for all compounds. The substituted haloindole-3-carboxyaldehydes (1.0 g) were dissolved in methanol (5 ml), and solid sodium borohydride was slowly added over a period of 30 min and

the reaction was continued until all the aldehyde was reduced as determined by thin-layer chromatography. The resulting solution was added to 50 ml of water, cooled to 0°C, and the halo-substituted indole-3-carbinols were collected by filtration and dried in a vacuum dessicator in the absence of light (yields 80–90%). This reaction and reaction products must be synthesized, isolated and stored in the dark due to the photosensitivity of the halo-substituted indole-3-carbinol products. These compounds (1.0 g) were then dissolved in 2–3 ml methanol, added to 80 ml of aqueous phosphate buffer (pH 5.5) and stirred at 20–25°C in the dark for 12–72 h, and reaction progress (i.e. formation of the less polar DIM condensation products) was monitored by thin-layer chromatography. The resulting substituted DIMs were filtered, dried in vacuo, and stored in the dark (yield 80–95%). Structures and purities of these compounds were confirmed by chromatographic methods and nuclear magnetic resonance spectroscopy. Three hundred MHz nuclear magnetic resonance spectra were determined in deuteroacetone to give the following: 5,5′-dichloroDIM, 4.20 (CH_2, s), 7.03 (H6, dd, J = 8.2, 2.1 Hz), 7.24 (H2, m), 7.65 (H7, d, J = 8.2 Hz); 7.53 (H4, d, J = 2.1 Hz), 10.09 (NH, s); 6,6′-dichloroDIM, 4.19 (CH_2, s), 6.94 (H5, dd, J = 8.2, 2.1 Hz), 7.16 (H2, m), 7.39 (H7, d, J = 2.1 Hz), 7.51 (H4, d, J = 8.2 Hz), 10.1 (NH, s); 4,4′-dichloroDIM, 4.7 (CH_2, s); 6.91–7.06 (H2, 5 and 6, m), 7.35 (H7, dd, J = 8.2, 2.0 Hz), 10.23 (NH, s); 5,5′-dibromoDIM, 4.20 (CH_2, s), 7.15 (H6, dd, J = 8.2, 2.1 Hz), 7.21 (H2, m), 7.34 (H7, d, J = 8.2 Hz), 7.69 (H4, d, J = 2.1 Hz), 10.19 (NH, s); 5,5′-dichloro-2,2′-dimethylDIM, 2.40 (CH_3, s), 4.04 (CH_2, s), 6.90 (H6, dd, J = 8.2, 2.1 Hz), 7.20–7.23 (H7 and 4, m), 10.01 (NH, s); 5,5′-difluoroDIM, 4.18 (CH_2, s), 6.83 (H6 and 2, m), 7.18–7.36 (H7 and 4, m), 10.04 ppm (NH, m), (note: all NH peaks were broad). Unlabeled TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were prepared in this laboratory (98–99%) as previously described. DIM was prepared by dimerization of indole-3-carbinol (I3C) and DIM/substituted DIMs were stored in the dark to avoid photoinduced decomposition. MCF-7 and T47D cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Fetal calf serum(FCS) was obtained from Intergen (Purchase, NY). Minimum essential medium (MEM) was purchased from Life Technologies (Grand Island, NY). DMBA, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, phosphate-buffered saline (PBS), acetyl-CoA, E2 and 100 × antibiotic/antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and biochemicals were the highest quality available from commercial sources.

2.2. Treatment of B6C3F1 mice

Twenty-one-day-old B6C3F1 female mice were purchased from Jackson Laboratories and housed 6–9 per cage with ad libitum access to food and water. Substituted DIM analogs were dissolved in corn oil with slight warming, and the total dose divided into three daily administrations. Animals were divided into three groups of six to eight animals each and dosed every 24 h on days 21, 22 and 23. The first group received vehicle control corn oil (50 μl) by gavage. The second group received 0.02 μg/day E2 (0.1 ml corn oil) by i.p. injection since this route of administration gave maximal E2-induced responses. The third group received 100 mg/kg of substituted DIM analog by gavage plus 0.02 μg/day E2 by i.p. injection. The doses of E2 were the minimal effective dose, which significantly induced the three uterine responses of interest. Animals were sacrificed by carbon dioxide asphyxiation and cervical dislocation 20 h after the last treatment, and uteri and liver removed. Uteri were quickly cleaned of connective tissue, weighed, nicked, and blotted, and then bisected into right and left halves with each half containing an entire uterine horn. Livers were perfused with HEGD [25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol (v/v)] buffer prior to removal for EROD analysis (data not shown).

2.3. Progesterone receptor binding assay PR

Half of the uterine bisections from each treatment group were pooled in an ice cold TESHMo (10 mM Tris–Cl, pH 7.4, 1.5 mM EDTA, 15 mM thioglycerol, 10 mM sodium molybdate) buffer, 1 ml/50 mg tissue. Uteri were homogenized with 3 × 8 s bursts using a Brinkman/Polytron tissue grinder. Samples were then centrifuged for 1 h at 105 000 × g, and the clear supernatant, constituting the cytosol, was carefully decanted and immediately used for competitive bind-

ing assays. Cytosolic fractions were incubated with 20 nM [^{3}H]R5020 in the presence or absence of 2 μM unlabeled R5020 at 4°C. Following an 8 h incubation, samples were placed on ice and treated with 0.1 volume dextran-coated charcoal (DCC) suspension (0.5% dextran:5% charcoal, wt./vol. in TESHMo) for 10 min. Samples were then centrifuged at 5000 *g* for 10 min, and the supernatant measured by liquid scintillation counting. PR levels were calculated assuming a 1:1 binding between PR and [^{3}H]R5020. Levels are reported in fmol per uterus.

2.4. Uterine peroxidase assay (UPO)

Uterine bisections from the different treatment groups were pooled and homogenized in 10 mM Tris–Cl buffer. Homogenates were centrifuged at 39 000 × *g* at 2°C for 45 min and the resulting pellet was washed and resuspended in 10 mM Tris–Cl buffer containing 0.5 M $CaCl_2$. Extracts were clarified by centrifugation for 45 min at 39 000 × *g* at 2°C. Uterine peroxidase activity of supernatant fractions was determined as described [23]. Each assay mixture (3.0 ml total) contained 13 mM guaiacol and 0.3 mM H_2O_2 in the extraction buffer. The reaction was started by addition of 1.0 ml of $CaCl_2$ extract. The initial rate (1 min) of guaiacol oxidation was monitored at $\lambda = 470$ nm on a Beckman spectrophotometer. An enzyme unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per min under the assay conditions described. Enzyme activity is expressed per uterus.

2.5. Rat mammary tumor studies

Female virgin Sprague–Dawley rats were purchased from Harlan (Houston, TX) and were treated on 54 days of age with 20 mg DMBA in 0.5 ml corn oil. Tumors developed 30–75 days after administration of DMBA; after initial formation of tumors (250–400 mm^3), rats were treated by oral gavage every other day with vehicle control corn oil (4 ml/kg) or substituted DIMs (5.0 or 1.0 mg/kg) dissolved in corn oil (4 ml/kg). Tumors were measured with calipers and tumor volumes were calculated by the formula (length × width × depth)/6π. On day 21 (2 days after the last dose), animals were sacrificed by asphyxiation and tumor and organ weights were determined as previously described [20,21]. Livers were perfused and hepatic microsomal EROD activity was assayed (data not shown). All measurements are expressed as mean ± standard error and each treatment group contained 8–10 animals. Significance was determined by Duncan's new multiple range test.

2.6. T47D and MCF-7 cell proliferation assay

T47D and MCF-7 cells were maintained in αMEM supplemented with sodium bicarbonate (2.2 g/l), gentamycin (2.5 ml/l), penicillin/streptomycin (10 000 units/l, 10 mg/ml) and 5% FBS. T47D cells were seeded in 6-well plates (50 000/well) in DME-F12 supplemented with 5% FBS treated with dextran-coated charcoal, 1.2 g/l $NaHCO_3$ and 10 ml/l antibiotic solution. MCF-7 cells were seeded in 6-well plates at a density of 75 000/well. After 24 h, the cells were treated with the appropriate chemicals (i.e. 1 nM E2, haloDIM alone or in combination with 1 nM E2) dissolved in DMSO to give a final DMSO concentration in the media of 0.2% (vol./vol.). The medium was changed and cells were redosed every 48 h. After 10 (MCF-7) or 14 (T47D) days, cells were trypsinized, harvested, centrifuged at 200 × *g* for 5 min at 4°C and resuspended in fresh medium. Cells were counted using a Coulter Z1 cell counter (Coulter Electronics, Hialeah, FL).

2.7. Statistics

All in vitro cell proliferation assays were carried out in triplicate and results are expressed as means ± SE. The data were analyzed by ANOVA and Scheffe's test.

3. Results

3.1. Inhibition of E2-induced proliferation in breast cancer cells

T47D and MCF-7 breast cancer cells were used for investigating the mitogenic and growth inhibitory activity of dihaloDIMs. The effects of dihaloDIMs alone and in combination with 1 nM E2 on proliferation of T47D human breast cancer cells are summarized in Fig. 2. At concentrations ≤10 μM, only one compound, 4,4′-dichloroDIM slightly induced cell proliferation at a concentration of 5 μM, whereas 10

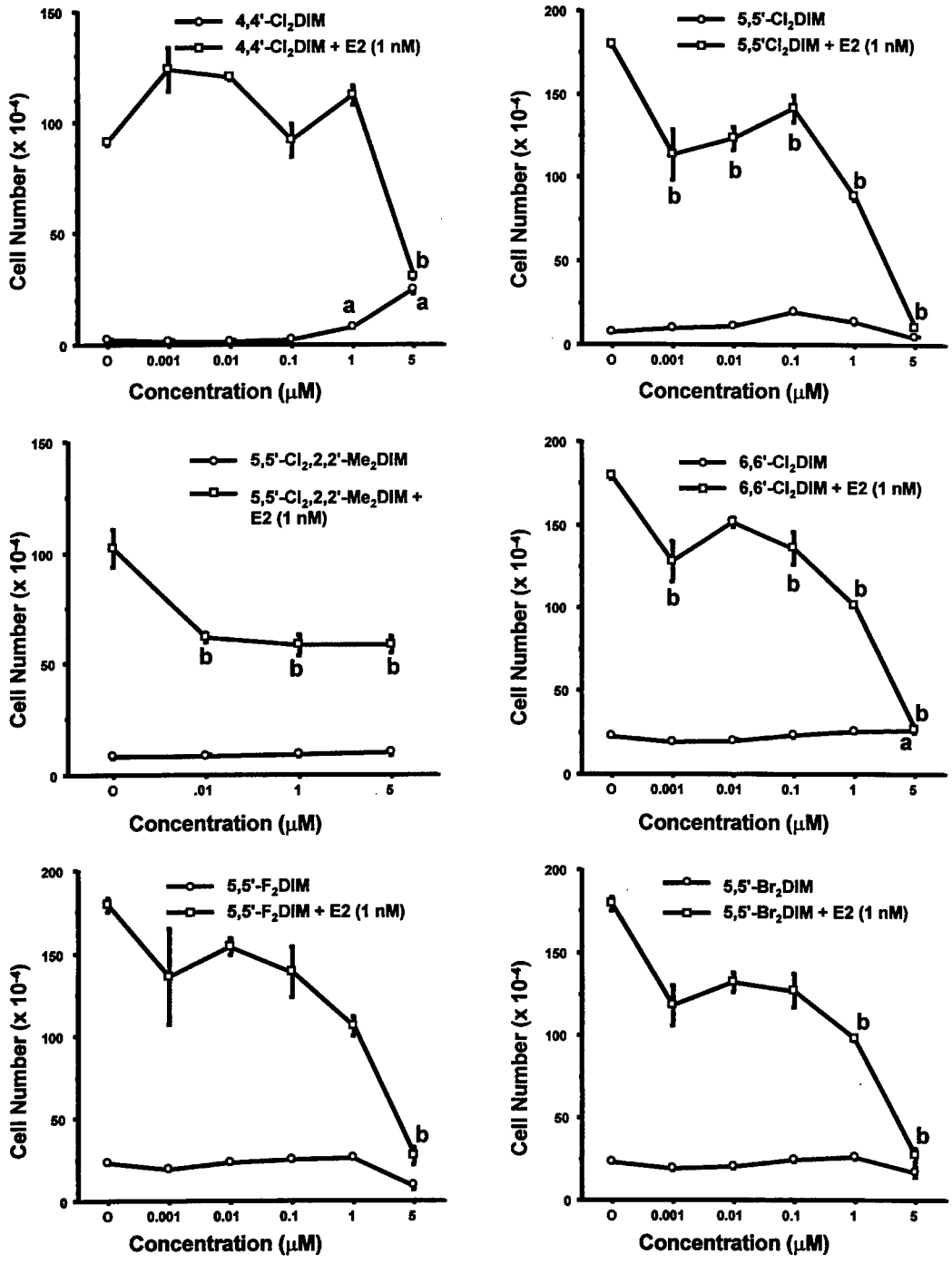

Fig. 2. Estrogenic and antiestrogenic activities of substituted DIMs in T47D cells. Effects of different concentrations of substituted DIMs alone or in combination with 1 nM E2 and cell proliferation was determined as described in the materials and methods. Results are expressed as means ± SE for at least three replicate experiments for each treatment group. Significant ($P < 0.05$) induction (a) of cell proliferation or inhibition (b) of estrogen-induced cell proliferation is noted.

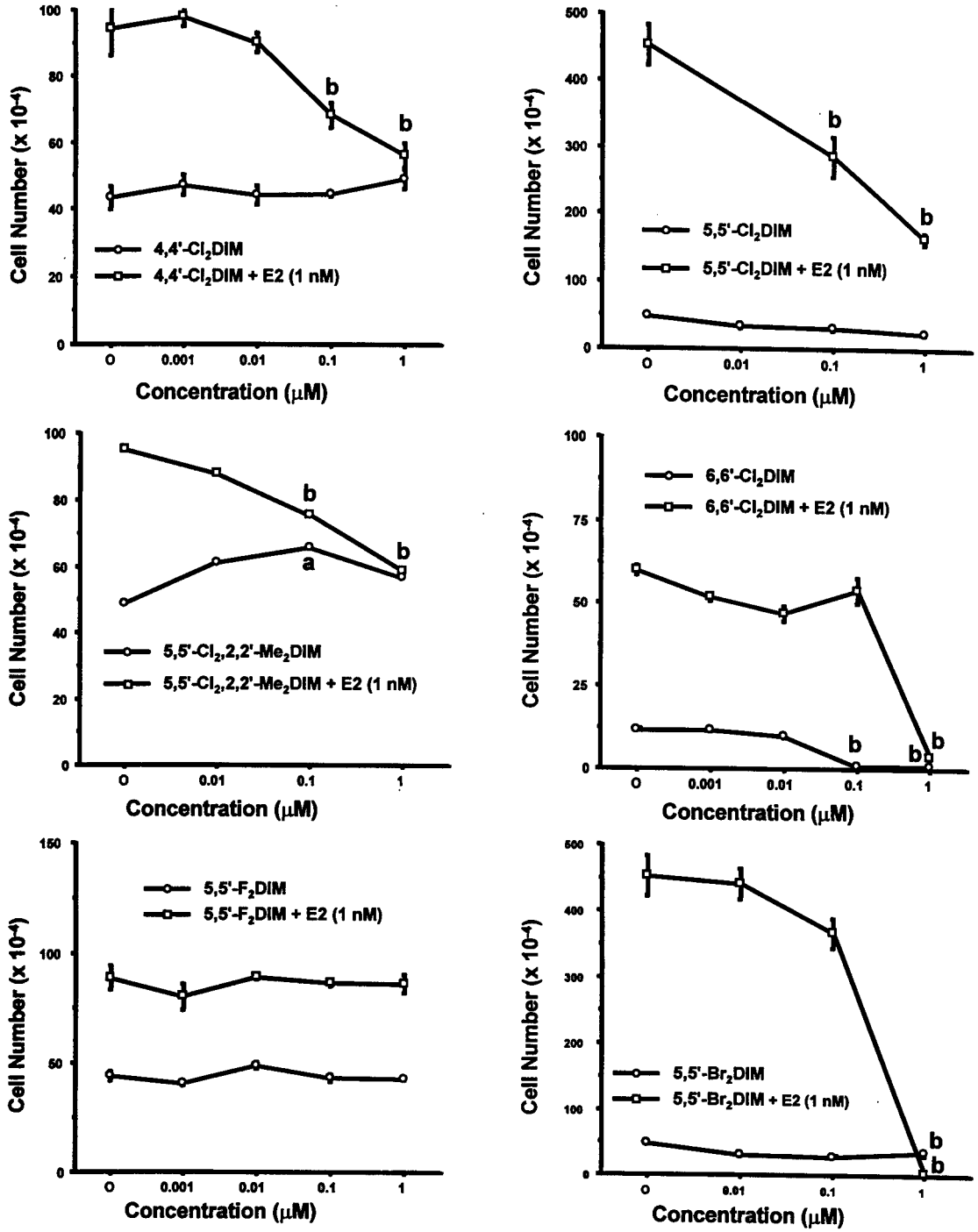

Fig. 3. Effects of dihaloDIMs on MCF-7 cells. Cells were treated with dihaloDIMs alone or in combination with E2 and proliferation was determined at each concentration as described in the materials and methods. Cytotoxicity was observed using 10 μM concentrations of dihaloDIMs (data not shown). Results are expressed as means ± SE for three separate determinations for each treatment group. Significant ($P < 0.05$) induction (a) of cell proliferation or inhibition (b) of estrogen-induced cell proliferation is noted.

μM 4,4′-dichloroDIM had no effect on cell growth and was not cytotoxic. The other dihaloDIMs were not mitogenic in T47D cells and this was consistent with their failure to bind the mouse uterine cytosolic ER (data not shown). Inhibition of estrogen induced growth of T47D cells by dihaloDIMs was biphasic; <50% growth inhibition was observed for most compounds at concentrations ≤1 μM and 90–100% growth inhibition was observed at concentrations of 5 or 10 μM. Only 5,5′-dichloro-2,2′-dimethylDIM slightly induced proliferation of MCF-7 cells, whereas the remaining compounds alone did not affect cell growth or were slightly growth inhibitory (Fig. 3). In cells co-treated with 1 nM E2 plus different concentrations of dihaloDIMs, the minimal inhibitory concentrations were 100, 100, 100, 1000 and 1000 nM for 5,5′-dichloro-, 5,5′dichlor-2,2′-dimethyl-, 4,4′dichloro-, 6,6′-dichloro-, and 5,5′-dibromoDIM, respectively, whereas no inhibitory responses were observed for 5,5′-difluoroDIM. The dihaloDIMs were cytotoxic to MCF-7 cells at concentration ≥10 μM, and results indicated that T47D cells were more sensitive than MCF-7 cells to inhibition of E2-induced proliferation. Inhibition of E2-induced proliferation of Ah-non-responsive benzo[a]pyrene-resistant MCF-7 cells by the dihaloDIMs, was also carried out as previously described [24], and only minimal inhibition (<30%) of E2-induced cell proliferation was observed (data not shown). These results are consistent with a role for the AhR in mediating inhibition of E2-induced proliferation of breast cancer cells but do not exclude other growth inhibitory pathways.

3.2. Estrogenic/antiestrogenic activities in the immature mouse uterus

The in vivo estrogenic and antiestrogenic activities of haloDIMs were initially investigated utilizing the immature mouse uterus, which is responsive to both estrogens and AhR-based antiestrogens [25,26]. The test compounds were dissolved in corn oil and administered by oral gavage to 21-day-old female B6C3F1 mice every 24 h for 3 days, and E2 was administered by i.p. injection. Twenty hours after the final dose, mice were sacrificed and three estrogen-responsive parameters were determined, namely uterine wet weight, uterine PR binding and uterine peroxidase activity (Table 1). At a dose of 100 mg/kg, estrogenic activity was not observed for any of the dihaloDIM analogs, whereas E2 (0.02 μg/mouse/day × 3) induced a significant 2- to 10-fold increase in uterine wet weight, PR binding and peroxidase activity. Compared to corn oil (control) treated animals, the only significant effects were observed for PR binding, which was decreased after treatment with 5,5′-difluoro-, 5,5′-dichloro-, 4,4′-dichloro- and 5,5′-dichloro-2,2′-dimethylDIM alone. Mice were then co-treated with E2 (0.02 μg/mouse/day) plus individual dihaloDIMs (100 mg/kg) for three consecutive days and estrogenic responses were determined. None of the compounds significantly inhibited E2-induced uterine wet weight; 5,5′-dichloro- and 6,6′-dichloro-DIM inhibited both E2-induced PR binding and peroxidase activity, whereas 5,5′-difluoro-, 5,5′-dichloro-2,2′-dimethyl-DIM and 5,5′-dibromoDIM inhibited only the latter response. Thus, inhibition of E2-induced uterine peroxidase activity was observed for five of the six dihaloDIMs, and this was the most sensitive indicator of antiestrogenic activity in the mouse uterus for these compounds.

3.3. Antitumorigenic activities of dihaloDIMs

Previous studies showed that DIM inhibited growth of DMBA-induced rat mammary tumors at a dose of 5 mg/kg every second day, whereas antitumorigenic responses were not observed at lower doses (1.0 or 0.5 mg/kg every second day) [21]. Therefore, the dihaloDIMs were investigated at a dose of 1.0 mg/kg to determine differences in antitumorigenic potency with the parent compound, DIM. Results summarized in Fig. 4, show that among the dihaloDIM analogs, 5,5′-dibromo-, 6,6′-dichloro- and 4,4′-dichloroDIM significantly inhibited tumor growth at a dose of 1.0 mg/kg, whereas the 5,5′-dichloro-2,2′-dimethyl-, 5,5′-dichloro- and 5,5′-difluoroDIM were inactive at the same dose. The effects of dihaloDIMs on tumor weight were similar to those observed for tumor volume (data not shown) and 4,4′-dichloro DIM was the most active compound in this study. Two of the inactive analogs (5,5′-dichloro- and 5,5′-difluoroDIM) were retested at a higher dose (5.0 mg/kg) and only 5,5′-difluoro-DIM was antitumorigenic at the higher dose. The dihaloDIMs did not significantly decrease body or organ weights and the only significant

Table 1
Effects of dihaloDIMs and E2 alone and in combination on uterine wet weight, peroxidase activity, and PR binding in the immature female B6C3F1 mouse[a]

Compound	Uterine wet wt. (mg) (% E2 act.)	PR (fmol/uterus) (% E2 act.)	Peroxidase activity (units/mg) (% E2 act.)
Corn oil	11.2 ± 1.8	270 ± 54	0.17 ± 0.00
E2 (0.02 μg/mouse)	31.8 ± 4.1[b]	1470 ± 154[b]	1.16 ± 0.07[b]
E2 + 4,4′-dichloroDIM	32.1 ± 5.9 (101)	1357 ± 50 (92)	1.24 ± 0.07 (107)
Corn oil	13.9 ± 3.1	200 ± 32	0.22 ± 0.01
E2 (0.02 μg/mouse)	33.5 ± 4.9[b]	1875 ± 137[b]	1.48 ± 0.01[b]
E2 + 5,5′-dichloroDIM	29.5 ± 2.1 (88)	710 ± 106 (38)[c]	0.87 ± 0.01 (59)[c]
Corn oil	12.1 ± 1.7	350 ± 47	0.19 ± 0.01
E2 (0.02 μg/mouse)	30.1 ± 3.3[b]	1568 ± 100	1.14 ± 0.04[b]
E2 + 6,6′-dichloroDIM	25.3 ± 2.0 (84)	1176 ± 58 (75)[b]	0.38 ± 0.02 (33)[c]
Corn oil	12.2 ± 3.1	198 ± 132	0.18 ± 0.02
E2 (0.02 μg/mouse)	45.9 ± 8.6[b]	1464 ± 119[b]	3.42 ± 0.02[b]
E2 + 5,5′-difluoroDIM	41.6 ± 5.6 (91)	1417 ± 94 (97)	2.79 ± 0.07 (82)[c]
Corn oil	12.9 ± 3.6	300 ± 2	0.17 ± 0.01
E2 (0.02 μg/mouse)	33.7 ± 5.1[b]	1170 ± 37[b]	1.42 ± 0.03[b]
E2 + 5,5′-dibromoDIM	34.6 ± 4.7 (103)	1759 ± 150 (150)	1.09 ± 0.05 (77)[c]
Corn oil	8.8 ± 1.3	260 ± 22	0.15 ± 0.01
E2 (0.02 μg/mouse)	35.0 ± 3.8[b]	1779 ± 123[b]	1.63 ± 0.01[b]
E2 + 5,5′-dichloro-2,2′-dimethylDIM	33.8 ± 3.0 (97)	1566 ± 163 (88)	1.23 ± 0.02 (75)[c]
Corn oil	10.7 ± 1.8 (32)	266 ± 32 (20)	0.12 ± 0.01 (8)
E2 (0.02 μg/mouse)	33.1 ± 6.3 (100)[b]	1317 ± 239 (100)[b]	1.43 ± 0.00 (100)[b]
5,5′-dibromoDIM	11.7 ± 3.0 (35)	169 ± 23 (13)	0.13 ± 0.01 (9)
5,5′-dichloroDIM	10.0 ± 1.8 (30)	12 ± 56 (1)	0.13 ± 0.01 (9)
5,5′-difluoroDIM	9.7 ± 1.4 (29)	20 ± 38 (2)	0.13 ± 0.01 (9)
6,6′-dichloroDIM	10.5 ± 2.2 (32)	166 ± 70 (13)	0.16 ± 0.01 (11)
4,4′-dichloroDIM	9.3 ± 1.5 (28)	74 ± 19 (6)	0.12 ± 0.01 (8)
5,5′-dichloro-2,2′-dimethylDIM	8.3 ± 1.3 (33)	47 ± 8 (4)	0.14 ± 0.08 (18)

[a] DihaloDIMs (100 mg/kg), E2 or their combination were administered to 21-day-old female B6C3F1 mice every 24 h (3 ×) and the uterine responses were determined 20 h after the third dose as outlined in the materials and methods. Hepatic CYP1A1-dependent EROD activity was induced <2-fold by the dihaloDIMs alone at the 100 mg/kg dose (× 3) (data not shown). ND, not determined.
[b] Significantly greater than control ($P < 0.05$).
[c] Significantly lower than E2-induced activity ($P < 0.05$).

response was an increase in kidney weight in animals treated with 5,5′-dichloro-2,2′-dimethylDIM (data not shown).

4. Discussion

I3C is a phytochemical found in cruciferous vegetables such as broccoli, Brussels sprouts and cauliflower and several in vivo studies show that I3C and cruciferous vegetables exhibit antitumorigenic activity [27–33]. I3C is both photo- and acid-labile, and at low pH, I3C polymerizes to multiple products [34] including DIM. I3C and/or DIM inhibit estrogen-induced responses in both in vivo and in vitro models and at a dose of 5.0 mg/kg every second day, DIM

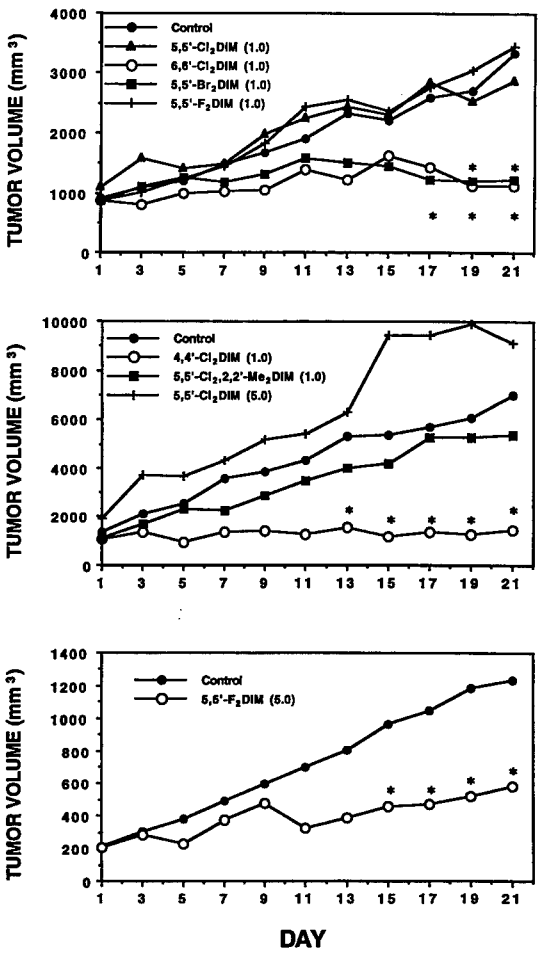

Fig. 4. Antitumorigenic activity of haloDIMs in female Sprague–Dawley Rats. After initial development of DMBA-induced mammary tumors, haloDIMs (1.0 or 5.0 mg/kg per 2 days) were administered by oral gavage and tumor growth was determined as described in the materials and methods. Results are expressed as means ± SE for 8–10 animals for each treatment group and significant decreases in tumor volume ($P < 0.05$) are indicated with an asterisk. Body and organ weights were not significantly affected by treatment with dihaloDIMs, and induction of hepatic CYP1A1-dependent EROD activity was not observed (data not shown).

inhibits growth of DMBA-induced mammary tumors in female Sprague–Dawley rats [21]. DIM is an AhR agonist and results of previous studies [21] are consistent with AhR-mediated antiestrogenic activity associated with inhibitory AhR–ER crosstalk. DIM, like alternate-substituted alkyl PCDFs, is relatively nontoxic and represents a new class of phytochemical-derived AhR-based antiestrogens with potential for clinical treatment of breast cancer in women. This study was designed to investigate the antiestrogenic and antitumorigenic activities of haloDIMs using both in vivo and in vitro models.

Like DIM [21], the dihaloDIMs exhibited weak AhR agonist activity and did not induce CYP1A1-dependent EROD activity in vivo or in vitro (data not shown). However, the antiestrogenic growth inhibitory activity of the dihaloDIMs observed in Ah-responsive MCF-7 cells (Fig. 3) was not observed in Ah-non-responsive benzo[a]pyrene-resistant MCF-7 cells (data not shown), suggesting that the antiestrogenic responses may, in part, be AhR-dependent. In T47D cells, the dihaloDIMs were more potent as inhibitors of E2-induced cell proliferation (Fig. 2), and these effects were biphasic: modest (<40%) inhibition of E2-induced cell proliferation was observed at concentrations from 0.01 to 1 μM, followed by >90% inhibition at higher concentrations (1–10 μM). These data are also consistent with more than one mechanism of growth inhibition that could include the AhR and other growth inhibitory pathways.

We have previously reported that other classes of AhR agonists inhibit estrogen-induced responses in the rodent uterus and mammary tumor growth in female Sprague–Dawley rats initiated with DMBA [20,21,24,25]. In the B6C3F1 mouse uterine assay the dihaloDIMs were not estrogenic at a dose of 100 mg/kg (×3); however, in co-treatment studies (E2 plus dihaloDIMs), one or more E2-induced uterine responses were inhibited. Interestingly, none of the compounds inhibited E2-induced uterine wet weight and these results were comparable to studies with other AhR agonists in which their antiestrogenic potencies were response-dependent and the uterotrophic effects of E2 were inhibited only at high doses [25].

Previous studies showed that DIM significantly inhibited growth of DMBA-induced mammary tumors at a dose of 5.0 mg/kg per 2 days but was inactive at lower doses (1.0 and 0.5 mg/kg per 2 days) [21]. Therefore, the effects of halogen substitutes on enhancing antitumorigenic activity were investigated at a dose (1.0 mg/kg per 2 days) that was inactive for DIM. Structure-antitumorigenic relationships (Fig. 4) showed that three of the six compounds were active at a dose of 1.0 mg/kg per 2 days and these were 4,4′-dichloro-, 5,5′-dibromo- and

6,6′-dichloroDIM. Interestingly, 5,5′-difluoro but not 5,5′-dichloroDIM was antitumorigenic at a dose of 5 mg/kg per 2 days.

In summary, this study has identified a group of dihaloDIMs that exhibit antitumorigenic activity at doses as low as 1.0 mg/kg per 2 days, and these antitumorigenic responses were not accompanied by overt signs of organ weight loss or tissue injury. Thus, dihaloDIMs represent a potent class of relatively nontoxic compounds that inhibit mammary tumor growth in rodents and resemble DIM, one of the chemoprotective compounds derived from I3C and cruciferous vegetables. Ongoing studies are investigating other DIM analogs for development as antitumorigenic agents for potential treatment of breast cancer in women.

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SOT ABSTRACT 1020 , 2000

METHYLENE-SUBSTITUTED 1,1′-DIMETHYLDIINDOLYLMETHANE ANALOGS AS INHIBITORS OF CARCINOGEN-INDUCED MAMMARY TUMOR GROWTH IN RODENTS. D Morrow, A McDougal and S Safe. Dept. of Veterinary Physiology & Pharmacology, Texas A&M University, College Station, TX, USA.

Diindolylmethane (DIM) is an aryl hydrocarbon receptor (AhR) agonist and inhibits estrogen-induced responses in the rodent uterus and carcinogen-induced mammary tumor growth in female Sprague-Dawley rats. A number of new methylene-substituted analogs of DIM have been synthesized and investigated for their antiestrogenic and antitumorigenic activities in rodent models. Initial studies examined 1,1′-dimethyldiindolylmethane analogs substituted at the CH_2 bridge with 4-hydroxyphenyl, 4-methoxyphenyl and 1-naphthyl substituents. All of these compounds were only weakly active in AhR binding or transformation assays, and at doses as high as 100 mg/kg/day (X3), no induction of hepatic microsomal CYP1A1-dependent ethoxyresorufin *O*-deethylase (EROD) activity was observed in C6B3F1 mice. Despite the apparent lack of AhR agonist activity, the C-substituted DIM analogs inhibited growth of 7,12-dimethylbenz[a]anthracene-induced mammary tumors at doses as low as 1.0 mg/kg/every second day. At this dose, there was no significant tumor growth throughout 21 days of treatment, whereas in vehicle control animals, tumor volumes increased >3.5-fold. Thus, these analogs are more potent inhibitors of mammary tumor growth than DIM, and preliminary results suggest that their mechanisms of antitumorigenic action are at least, in part, through AhR-independent pathways.

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